# METHOD AND DEVICE FOR THE DETECTION OF MUTATIONS IN ISOLATED GENE SEQUENCES OF THE LOW DENSITY LIPOPROTEIN RECEPTOR (LDL-R) WHICH IS ASSOCIATED WITH WITH FAMILIAL HYPERCHOLESTEROLEMIA

## Field of the invention

The present invention falls within the technical-diagnostic extra-corporeal "in vitro" biological samples sector, for determining an individual's predisposition to the disease named Familial Hypercholesterolemia.

## Background of the invention

According to the WHO definition, atherosclerosis is a combination of changes in the intimae of the arteries resulting from focal accumulation of lipids and complex compounds, accompanied by fibrous tissue formation, calcification and in turn associated with changes in the media.

Atherosclerosis may be considered as a special form of arteriosclerosis with pathogenic significant deposition of lipids in the arterial wall. Most forms of arteriosclerosis involve fatty degeneration of vascular wall, the terms "arteriosclerosis" and "atherosclerosis" may be used synonymously (Assmann G. in "Lipid Metabolism and Atherosclerosis" Schattauer Verlag GMbH, Stuttgart 1982:1).

Lipids are insoluble in aqueous solutions. Lipoproteins are the particles enabling transport of the lipids in the blood. Lipoproteins are divided into various categories according to their density, depending on how they can be separated by ultracentrifugation (Havel RJ et al. J Clin Invest 1955, 34:1345). Low density-lipoproteins (LDL) (d=1.019-1.063 g/mL) transport the bulk of the cholesterol in the blood. They are composed of about 75% lipid (primarily cholesterol, cholesteryl esters and phospholipids), approximately 70% of the total cholesterol in the blood is transported by LDL particles.

Hypercholesterolemia is used to reflect a rise in plasma cholesterol higher than level considered normal for a particular poupulation and is one of the critical factors in the onset and the progress of atherosclerosis. More than half of all deaths in Western

society are related to atherosclerosis cardiovascular diseases (Murray CJL and Lopez AD. Lancet 1997; 349:1269-1276).

Familial hypercholesterolemia (FH) is an autosomal dominant inherited disease produced in the receptor gene of the LDL (LDL-r) this gene codifies a protein that allows the intracellular uptake and degradation of the LDL (Goldstein JL, Brown MS Ann Rev Cell Biol 1985; 1:1-39).

The penetrance of FH is almost 100% meaning that half of the offspring of an affected parent has a severely elevated plasma cholesterol level from birth onwards, with males and females equally affected (Goldstein JL, Brown MS. The metabolic basis of inherited disease. Scriver CR, Beaudet AL, Sly WS, Valle D, eds. McGraw Hill New York 6$^{th}$ edition, 1989; 1215-1250).

FH affected individuals display arcus lipoides corneae, tendon xanthomas and premature symptomatic coronary heart disease (Scientific Steering Committee on behalf of the Simon Broome register Group. Atherosclerosis 1999; 142: 105-115). FH is one of the most common inherited disorders with frequencies of heterozygote patients of and homozygote estimated to be 1/500 and 1/1,000,000, respectively.

Certain populations, such as a small number of mutations predominate due to founder effects and therefore, the frequency of heterozygous FH is higher, these populations include French Canadians (Leitersdorf E et al. J Clin Invest 1990; 85:1014-1023), Christian Lebanese (Lehrman MA et al. J Biol Chem 1987; 262:401-410) Druze (Landsberger D et al. Am J Hum Genet 1992; 50: 427-433) Finns (Koivisto UM et al. J Clin Invest 1992; 90:219-228) South African Afrikaner (Kotze MJ et al. Ann Hum Genet 1991; 55:115-121), and Ashkenazi Jews of Lithuanian descent (Meiner V et al. Am J Hum Genet 1991; 49:443-449) have the peculiarity that they have only a few mutations responsible for the FH, result of founder effects and therefore the frequency of heterozygous FH in those populations is higher than the estimate for other populations.

FH heterozygous patients display a very high plasma cholesterol concentration, generally above the 95$^{th}$ percentile value. In patients with FH the age-standardised and sex-standardised mortality ratios are four to five times higher than in the general population (Scientific Steering Committee on behalf of the Simon Broome Register Group. Atherosclerosis1999; 142: 105-115). Patients who have inherited two mutant at the LDL-r locus are termed "FH homozygotes" or "FH compound heterozygotes", in

which case those are practically no functional receptors which lead to a six-fold to eight-fold elevation in plasma LDL-c levels above normal. In the majority of these patients, a coronary heart disease typically occurs before the age of 20 years (Goldstein JL et al. N Engl J Med 1983; 309:288-296). If individuals with heterozygous or homozygous FH could be diagnosed before they develop symptomatic disease, they could be treated preventively to substantially reduce their risk of myocardial infarction.

The LDL-r is an ubiquitous trans-membrane glycoprotein of 839 amino acids that mediates the transport of LDL into cells via endocytosis (Goldstein J and Brown M J Biol Chem 1974; 249:5153-5162) (Figure 1).

The LDL-r gene lies on the short arm of chromosome 19p13.1-13.3 (Yamamoto T et al. Cell 1984;39: 27-38), spans 45,000 base pairs (bp). It comprises 18 exons and 17 introns encoding the six functional domains of the mature protein: Signal peptide, ligand-binding domain, epidermal growth factor (EGF) precursor like, O-linked sugar, trans-membrane and cytoplasmic domain (Sundhof T et al. Science 1985; 228:893-895) (Figure 2).

The LDL-r synthesis is regulated by a sophisticated feedback mechanism that controls the transcription of the LDL-r gene in response to variations in the intracellular sterol concentration and the cellular demand for cholesterol (Sudhof TC et al J Biol Chem 1987; 262:10773-10779). DNA motifs necessary for transcriptional regulation of the LDL-r gene are located within 177 bp of the proximal promoter (Sudhof TC et al. J Biol Chem 1987; 262: 10773-10779). This region contains all the cis-acting elements for basal expression and sterol regulation and includes three imperfect direct repeats of 16 bp each. Repeat 1 and 3 containing binding sites for the transcriptional factor Sp1 and are essential for producing the basal expression of the gene but require the contribution of the repeat 2 for full expression (Dawson PA et al. J Biol Chem 1988; 263;3372-3379). Repeat 2 contains a 10 bp regulatory element , SRE-1, (Smith JR et al. J Biol Chem 1990; 265:2306-2310) that allows binding of the transcriptional factor designated as SREBP-1, when the intra-cellular sterol concentration diminishes. To date, several naturally-occurring mutations have been mapped to the transcriptional regulatory elements of the LDL gene receptor (Hobbs HH, et al. Hum Mutat 1992; 1:445-466; Koivisto UM, et al ProcNatl Acad Sci USA, 1994; 91:10526-10530), Mozas P, et al J Lipid Res 2002; 43:13-18, http.//www.ucl.ac.uk/fh; http.//www.umd.necker.fr).

Exon 1 encodes the signal peptide, a sequence of 21 amino acids, which is cleaved from the protein during the translocation into the endoplasmic reticulum. Several frameshift, missense and nonsense mutation has been described in this exon (http.//www.ucl.ac.uk/fh; http.//www.umd.necker.fr)

Exons 2 to 6 encode the ligand binding domain, which consists of seven tandem repeats of 40 amino acids each. The structure of the ligand binding domain has been partially elucidated (Jeon H et al. Nature Struc Biol 2001; 8:499-5049). There are a cluster of negatively charged amino acids, Asp-X-Ser-Asp-Glu in each repeat and six cysteine residues that form three disulfide bonds.

The second domain of the human LDL-r consists of 400 amino acid sequence, encoded by exons 7 to 14. This sequence shows a 33% of homology of the epidermal growth factor precursor (EGFP). Like the ligand binding domain, this region also contains three repeats of 40 amino acids with cysteine-rich sequences. The first two repeats, designated A and B, are contiguous and separated from the third repeat, by a 280 amino acid region that contains five copies of the YWTD (Tyr-Trp-Thr-Asp) sequence. The EGFP like domain is fundamental for the acid–dependent dissociation of the LDL particles from the LDL-r and clathrin coat pits that takes place in the endosome during receptor recycling. Of the all mutations described to date, approximately 55% are located in the EGFP-homology region and 35% among the YWTD repeats (http.//www.ucl.ac.uk/fh).

The third domain of the LDL-r that is encoded by exon 15, is a region rich in threonine and serine residues,. The function of this domain is unknown, but it is known that in this region the carbohydrate chains are anchored. This region show minimal sequence conservation among six species analysed and it is thought that this domain play a role in the stabilization of the receptor (Goldstein et al. In The Metabolic and Molecular Basis of Inherited Disease. Sciver CR, Beaudet AL, Sly WS, Valle D. 7$^{th}$ Edition. McGraw Hill, 1995: 1981-2030).

The trans-membrane domain comprises 22 hydrophobic amino acids coded by exon 16 and the 5′end of exon 17. This domain is essential for anchoring the LDL-r to the cell membrane.

The cytoplasmic domain of the LDL-r, is formed by a sequence of 50 amino acids residues, is encoded by the 3′ region of the exon 17 and the 5′ end of the exon 18. This

domain contains two sequence signals for targeting the protein to the cell surface and for localizing the receptor in coated pits (Yokode M, et al. J Cell Biol 1992;117: 39-46). This domain is one of the most conservedm with a percentage of amino acids converved of 86 % among six species analysed.

LDL-r mutations found in FH patients, have been classified into 5 classes: null alleles, transport defective alleles, binding defective alleles, internalization-defective alleles and recycling-defective alleles. As a general rule, each category is associated with mutations localised in a region of the gene that codes for one particular domain of the protein (Hobbs, HH, et al. Hum Mutat 1992; 1:445-466).

The heterogeneity in FH patients in relation to plasma LDL-c levels and coronary heart disease is due in part to differences in the nature of the mutation (Sun XM et al. Arterioscler Thromb Vas Biol 1993; 13:1680-1688, Kotze MJ et al. Arterioscler Thromb Vas Biol 1993; 13:1460-1468; Gudnason V et al. Arterioscler Thromb Vas Biol 1997;17:3092-3101). On the other hand, in FH heterozygote patients, the LDL-c lowering response after treatment with hydroxy-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors depends in part on the nature of the mutation in the LDL-r gene (Leisterdorf E et al. Circulation 1993; 87:35-44; Jeenah M et al. Atherosclerosis 1993; 98:51-58, Sijbrands EJG et al. Atherosclerosis 1998;136: 247-254).

The primary ligand for the receptor is LDL, which contains a single copy of a protein called apolipoprotein B-100 (ApoB-100) (Goldstein J and Brown M J Biol Chem 1974;249:5153-5162). This apolipoprotein has a zone rich in basic amino acids and being the site where binding to the receptor (Borén J et al. J Clin Inves 1998; 101: 1084-1093). Several mutations located in the apolipoprotein B gene have been found altering the functional activity of the protein and decreasing its capacity for withdrawal LDL particles, and leading to accumulation of LDL cholesterol in plasma. To date, four mutations have been identified in the apo B-100 gene which cause a hypercholesterolemia named Familial Defective (BDF) apolipoprotein, all of them located in the LDL-r binding domain of the apo B-100 protein (residues 3130-3630): R3480W, R3500Q, R3500W and R3531C (Soria L et al. Proc Natl Acad Sci USA 1989; 86: 587-591; Pullinger CR,et al. J Clin Invest 1995; 95:1225-1234; Gaffney D, et al. Arterioscler Thromb Vasc Biol 1995; 15:1025-1029; Boren J, et al. J Biol Chem 2001; 276;9214-9218). The CGG-to-CAG mutation at codon for amino acid 3500, resulting in a

glutamine substitution for arginine (R3500Q), is the most frequent alteration causing Familial Defective apolipoprotein B-100 (FDB). Patients heterozygous for the apoB-3500 mutation are usually hypercholesterolemic, although serum cholesterol concentrations can vary from those found in FH to only modest elevations (Tybjaerg-Hansen A, et al. Atherosclerosis 1990; 80:235-242; Hansen PS, et al. Arterioscl Throm Vasc Biol 1997; 17:741-747). Since clinical and biochemical characteristics in those patients are very similar, the differential diagnostic between patients with FDB or FH is only possible by genetic molecular diagnosis.

The clinical diagnosis of FH is based on the analytical data of lipids and lipoproteins in the plasma, clinical symtomatology (xanthomas) and family and personal coronary disease history. The WHO, through its MedPed program recommends a series of criteria be followed to perform the clinical diagnosis of FH. These criteria are based on a scoring system relying on the personal and family history of hypercholesterolemia, of the patient's clinical and analytic characteristics. When the punctuation reached by the patient is equal to or higher than 8 points the clinical criterion of FH diagnosis is classed as "certain", between 5 and 8 points as "likely· and between 3 and 5 points as "possible" (Familial Hypercholesterolemia. Report of a second WHO consultation. The International MedPed FH Organization, Geneva 1998). However, some patients do not fulfil the FH criteria, because the family history is incomplete or unknown, or because at the time of the analysis they presented only moderate concentrations of plasmatic cholesterol and lacked signs of tissue cholesterol deposition, as tendinosous xanthomas, arcus corneae or xanthelasmas.

In families whose mutation of the r-LDL gene is known, it has been demonstrated that the best "cut-off" point for the diagnosis is use of the 90th percentile for the c-LDL concentration (Umans-Eckenhausen MAW et al. Lancet 2001; 357:165-168. However, 18% of FH patients carriers of the mutuation have a total cholesterol concentration below this percentile and moreover the proportion of false positives was 18%. Therefore, there will be a high percentage of wrong diagnoses if only the plasmatic cholesterol figure is utilized. It has been published that more than 50% of patients do not receive lipid lowering therapy and dietary counselling as a result of not having been diagnosed correctly as patients with FH (Williams RR et al., Am J Cardiol. 1993; 72:18D-24D).

The elucidation of the molecular basis of FH has made diagnosis at the DNA level feasible in the vast majority of cases. Demonstration of an underlying defect in the LDL-r gene, constitutes in fact the definite confirmation of the diagnosis (Familial hypercholesterolemia. Report of a second WHO consultation. The International MedPed FH Organization, Geneva 1998). Although accurate diagnosis of FH is possible by means of molecular methods, their use in heterogeneous populations is limited at present owing to mutational heterogeneity of the LDL-r gene.

In application PCT WO-88/03175 (Biotechnology Research Partners, Ltd.) a method is claimed for the diagnosis of atherosclerosis, based on detection of the presence or absence of various polymorphisms in the gene region of the apolipoprotein AO-CIII-AIV, or in the genes apoB, apoCI, apoAII, as well as in the LDL receptor gene. Specifically for this gene, utilization of the polymorphisms Cfr131 and BstEII is presented.

Another document of interest is Japanese patent JP-10099099 which refers to the use of a mutation in the codifier triplet of the amino acid 109, specifically the insertion of a C, for the diagnosis of abnormalities in the LDL receptor gene, although familial hypercholesterolemia is not specifically mentioned.

Finally, U.S. Patents US-4.745.060 and US-4.966.837, both of the University of Texas, present methods for the diagnosis of familial hypercolesterolemia on the basis of mutations in the LDL receptor gene. However, what is claimed in the first of them are sequences corresponding to the "normal" gene, presenting a particular example of a mutation that is defined by the restriction map change with Xba I. In the second patent, on its part, the use of various restriction enzymes is claimed (Eco RI, Asp 718, Taq I, Bam HI, Xba I, Inf.I, Bgl II, Cla I, Eco RV, Kpn I, Pvu II, Sph I, Sst I, Sst II, Stu I, Xho I, Nde I and Nsi I) in a method for determining mutations in the LDL-r gene, based on observing the alteration of the restriction model with these enzymes compared to the model corresponding to the normal gene.

The closest patent document to the invention is WO02/06467, in which a method is described, for the detection of errors in the lipidic metabolism based on a series of mutations and polymorphisms of the LDL-r gene. However, none of the mutations or polymorphisms described in said patent coincides with those claimed in the present application.

**Detailed description of the invention**

The nomenclature of the mutations and polymorphisms is defined in

- Antoranakis S. E. and the Nomenclature Working Group, Recommendations for Nomenclature Systems for Human Gene Mutations. Human Mutation 11:1-3; 1998
- Dunnen JT, Antoranakis S.E. Mutation Nomenclature Extensions and Suggestions to describe Complex Mutations: A Discussion. Human Mutation 15: 7-12, 2000.

Similarly the concept of the polymorphisms is defined in

- Harris H. The Principles of Human Biochemical Genetics 3rd Edition. Amsterdam. North-Holland 1980.
- Beauder AL, Scriver CL, Sly WS, Valle D. Genetics, Biochemistry and Molecular Basis of Variant Human Phenotypes, in The Metabolic and Molecular Bases of Inherited Disease. Editores Beaudet AL, Scriver CR, Sly WS, Valle D 7th Edition. Page 53, MacGraw Hill. New York 1995.

There has been detected, isolated and characterized a whole series of new mutations which are detailed below. Similarly, a whole series of mutations and polymorphisms already described, have combined with them to analyze the likelihood of an individual developing familial hypercholesterolemia. All of the mutations and polymorphisms which in this invention relate to development of familial hypercholesterolemia are produced in the gene sequence SEQ ID NO:1 corresponding to the low density lipoproteins receptor gene (LDL-r). That is to say, all of the mutations are produced in the same gene, are used in the same testing device, using the same technology to determine, using the same method, extra-corporeally and in vitro, the likelihood of developing the same disease, which supports the unitary nature of the invention.

In Table I all of the new mutations detected are detailed, according to the nomenclature scientifically approved and detailed in the publications mentioned above. Likewise, they are provided with an alpha-numerical code.

In Table II mutations are detailed, already described and known, whose use in combination with the mutations of Table I, in testing devices in vitro for diagnosis of the familial hypercholesterolemia is one of the preferred forms, new and inventive, of embodiment of the invention. Similarly, in analogous manner to that mentioned for the known mutations, in Table III polymorphisms are detailed.

The amino acid mutations are represented in one-letter codes which have their equivalence according to Table IV.

**TABLE I**

| MUTATION | ID |
|---|---|
| (-23)A>C | M002 |
| 1054 del11 | M006 |
| 108delC | M008 |
| 1197del9 | M009 |
| 1207delT | M010 |
| 1432delG | M012 |
| 191-2delAinsCT | M016 |
| 2184delG | M020 |
| 231delC | M022 |
| 2399del5/ins4 | M024 |
| 313+1insT | M027 |
| 338del16 | M029 |
| 509insC | M030 |
| 675del15 | M032 |
| 684dup12 | M034 |
| 941-39C>T | M041 |
| C195R | M046 |
| C255G | M0100 |
| C319Y | M050 |
| D157G | M059 |
| D630N | M063 |
| E291X | M068 |
| H635N | M096 |
| N59K | M074 |
| T41M | M097 |
| W515X | M098 |

| | |
|---|---|
| Y379X | M092 |
| Y421X | M093 |
| T433N | M105 |
| 818del8 | M110 |
| 1423delGC/insA | M111 |
| 1204insT | M112 |
| 451del3 | M115 |
| G516X | M117 |
| 2389+4A>G | M120 |
| 1815del11 | M121 |
| 1186+5G>A | M129 |
| T740M | M131 |
| I771T | M135 |
| R279G | M138 |
| T446I | M141 |
| H562Q | M142 |
| C74Y | M145 |
| D686Y | M147 |
| G(-2)R | M149 |
| E579D | M150 |
| S205C | M151 |
| D200V | M153 |
| V766E | M154 |
| L(-6)P | M155 |
| 2544insC | M156 |
| C42Y | M157 |
| 2389+3A>C | M160 |
| [1587-5del5;1587del31] | M161 |

**TABLE II**

| MUTATION | ID | MUTATION | ID |
|---|---|---|---|
| 2393del 9 | M001 | C646Y | M053 |
| (-42)C>G | M003 | C677Y | M054 |
| (-49)C>T | M004 | C68W | M055 |
| 1045delC | M005 | C74G | M056 |
| 1061-8T>C | M007 | C95R | M057 |
| A378T | M0102 | D151N | M058 |
| C358R | M0104 | D200G | M060 |
| 1358+1G>A | M011 | D200Y | M061 |
| 1706-10G>A | M014 | D280G | M062 |
| 1845+1G>C | M015 | E10X | M064 |
| 2085del19 | M017 | E246A | M066 |
| 211del G | M018 | E256K | M067 |
| 2140+5G>A | M019 | F634L | M069 |
| 2207insT | M021 | G322S | M070 |
| 2390-1G>C | M023 | G352D | M071 |
| 313+1G>C | M025 | G571E | M072 |

| | | | | |
|---|---|---|---|---|
| | 313+1G>A | M026 | N543H | M073 |
| | 313+2insT | M028 | N804K | M075 |
| | 518 del G | M031 | Q12X | M076 |
| | 7delC | M035 | Q133X | M077 |
| 5 | 872delC | M036 | Q357P | M078 |
| | 884delT | M038 | Q427X | M079 |
| | 920ins4 | M039 | Q71E | M080 |
| | A519T | M042 | R395Q | M081 |
| | C113W | M043 | R574W | M082 |
| 10 | C127R | M045 | R612C | M083 |
| | C255X | M047 | S156L | M084 |
| | C281Y | M048 | S205P | M085 |
| | C297F | M049 | T413K | M086 |
| | C347Y | M051 | T705I | M087 |
| 15 | C371X | M052 | V502M | M089 |
| | W(-18)X | M090 | | |
| | W541X | M091 | | |
| | D679E | M094 | | |
| | 1359-1G>A | M099 | | |
| 20 | 681ins21 | M033 | | |
| | C122X | M044 | | |
| | V408M | M088 | | |
| | G528D | M106 | | |
| | D412H | M107 | | |
| 25 | N619N | M108 | | |
| | E80K | M109 | | |
| | L534P | M113 | | |
| | L621S | M114 | | |
| | C356Y | M116 | | |
| 30 | R329X | M119 | | |
| | G248D | M122 | | |
| | C201Y | M125 | | |
| | 313+5G>A | M126 | | |
| | C358Y | M127 | | |
| 35 | C331R | M128 | | |
| | D157N | M130 | | |
| | V776M | M134 | | |
| | P664L | M136 | | |
| | W462X | M137 | | |
| 40 | Q328X | M139 | | |
| | L584P | M140 | | |
| | R395W | M143 | | |
| | G314V | M144 | | |
| | W469X | M146 | | |
| 45 | P678L | M148 | | |
| | R612H | M152 | | |
| | R236W | M159 | | |

**TABLE III**

| POLYMORPHISMS | ID |
|---|---|
| 81T>C BstUI Exón 2 | P1 |
| 1060+10G>C SmaI Exón 7 | P2 |
| 1171G>A StuI Exón 8 | P3 |
| 1413G>A DdeI Exón 10 | P4 |
| 1617C>T BstNI Exón 11 | P5 |
| 1725C>T SSCP Exón 12 | P6 |
| 1771C>T HincII Exón 12 | P7 |
| 1959 T>C AvaII Exón 13 | P8 |
| 2232G>A MspI Exón 15 | P9 |

**TABLE IV**

**AMINOACID NOMENCLATURE**

| | | |
|---|---|---|
| Alanine | Ala | A |
| Aspartic acid | Asp | D |
| Glutamic acid | Glu | E |
| Glycine | Gly | G |
| Phenylalanine | Phe | F |
| Leucine | Leu | L |
| Serine | Ser | S |
| Tyrosine | Tyr | Y |
| Cysteine | Cys | C |
| Tryptophan | Trp | W |
| Leucine | Leu | L |
| Proline | Pro | P |
| Histidine | His | H |
| Glutamine | Gln | Q |
| Arginine | Arg | R |
| Isoleucine | Ile | I |
| Methionine | Met | M |
| Threonine | Thr | T |
| Asparagine | Asn | N |
| Lysine | Lys | K |
| Serine | Ser | S |
| Arginine | Arg | R |
| Valine | Val | V |
| Stop codon | Ter | X |

The diagnosis assay "biochip" developed in the invention is a slide with a large amount of probes onto its surface shown in the sequences list. These oligonucleotide probes are able to hybridize with the mutant sequences included in Tables I to III. The methodology involved for each mutation is:

**Printing of glass slides**

- The oligonucleotides capable of detecting the mutation are printed onto an aminosilanized glass slide using DMSO as printing buffer.
- Printing is carried out with a "spotter" or oligonucleotides printer wherein temperature and humidity are controlled.

**Processing of the glass slides**

- After printing the slides undergo treatment with UV radition.

**Target-DNA preparation**

- Genomic DNA of the patient is extracted from a blood sample of approximately 300 µl by using a filtration method.
- A multiplex-PCR reaction is performed allowing amplification for each patient of the promoter and all 18 exons of the LDL receptor gene
- A biotinylated nucleotide is incorporated during the PCR process. As an indirect labeling method, a final step of staining with a fluorophore-streptavidin coupler is required after hybridization.
- PCR products are electrophoresed and visualized in agarose gel.
- Target-DNA is fragmented.
- Hybridization buffer is added to the fragmented PCR products.
- Denaturation step takes place at 95°C 15 min.

**Hybridization**

- Hybridization is carried out automatically in the station designed by Amersham Biosciences for this purpose.
- The glass slide is prehybridized.
- The hybridization solution is injected with a Hamilton pipelet.
- One hour is the hybridization time.
- The glass slide is washed three times and dried.
- The station dres the glass slide

**Scanning of glass slide**

- The glass slide in inserted in the scanner.
- The signal is scanned emitted by the standard marker on being stimulated by the laser.

**Quantification of image**

- The scanner software allows us to quantify in the image obtained the signal of the points where hybridisation has occurred.

- From the signal obtained in the oligonucleotides which detect the normal allele and the mutated one we establish the presence or absence of the mutation in the patient.

Each mutation has in the glass slide four oligonucleotides repeated 10 times for their detection. Two of them detect the normal allele and another two the mutated. The interrogated base is to be found in central position throughout.

In the case of a normal patient (Fig. 3A), he does not present mutated allele. Therefore, in the image obtained from the glass slide the oligonucleotides that detect said allele do not show hybridisation signal or a lesser signal than the oligonucleotides that detect the normal allele.

On the contrary, a heterozygous individual (Fig. 3B) for the mutation has the normal allele and the mutated one. Hence, the oligonucleotides which detect the normal allele and the mutated one have an equivalent hybridisation signal.

The results of the hybridisation of the DNA-chip with marked PCRs, produced from the DNA of the individuals to be analysed, demonstrate that the individual represented in Figure 3A does not have a particular mutation in the LDL-r gene which occasions a change of E256K amino acid, and that the individual of Figure 3B is heterozygous for this mutation.

In this way the heterozygous individual would be diagnosed genetically as Familial Hypercholesterolemic.

By means of analysis examples some of the mutations are next detailed, detected with the assay device of the invention.

**EXAMPLE 1: Identification of mutations located in exon 1 of the LDLr gene.**

A 215 bp fragment of exon 1 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the primers Ex1F (SEQ ID NO: 2) y Ex1R (SEQ ID NO: 3).

DNA (500ng) was amplified in a 50 µL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 µM each dNTP, 0.2 µM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 74°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by using the device described "biochip".

**(-23)A>C mutation analysis**

This mutation creates a new Ava II recognition site. Five microliters of the exon 1 amplied material were hydrolized with 15 units of Ava II in a total volume of 30 µL according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length of 148 and 67 bp for normal alleles and 93, 67 and 55 bp for mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide gel and were visualised by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38 and SEQ ID NO: 39.

(-23)A>C mutation was detected in a 60 years old woman with arcus corneae and xanthelasmas having been diagnosed as having familial hypercholesterolemia with a diagnostic score of 8 points in line with the MedPed (Familial Hypercholesterolemia. Report of a second WHO consultation. The International MedPed FH Organization, Geneva 1998). No evidence of premature cardiovascular event was detected in near relation with her family. The plasmatic concentration of lipids before the pharmacological treatment were: Total cholesterol (TC) 352 mg/dL, LDL-c 271 mg/dL, and the triglycerides (TG) and cholesterol of the high density lipoproteins (HDL-c) were within the normal range. Hypolypemiant treatment with simvastatin (20 mg/da) lowered her TC and LDL-c levels to 251 and 171 mg/dL respectively.

**L(-6)P mutation analysis**

This mutation (47T>C, CTC>CCC, Leu(-6)Pro) was characterized by automatic sequencing of the 215 bp fragment corresponding to exon 1 of the LDL-r gene on analysing this fragment clinically diagnosed as FH. The sequencing reaction was carried out in a PE Gene Amp System 9700 thermocyclator using the reagents of the CET 2000 Dye Terminator Cycle Sequencing kit with Beckman's Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex1F (SEQ ID NO:2) and Ex1R (SEQ ID NO:3). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL NDA Beckman Analysis System. The change T>C observed was confirmed by automatic sequencing of a second PCR product of the same sample.

Alternatively, this mutation can be analysed with the device described ("biochip") using in the slide the oligonucleotides SEQ ID NO:240, SEQ ID NO:241, SEQ ID NO:242 and SEQ ID NO: 243.

The L(-6)P mutation was detected in a 47 years old woman with arcurs corneae whose father had hypercholesterolemia with a TC of 350 mg/dL and two paternal uncles with hypercholesterolemia had died of myocardium attack at the age of 24 and 33 respectively. The clinical diagnosis of hypercholesterolemia familiar reached a score of 9 points according to MedPed criteria. The plasmatic concentrations of lipids prior to pharmacological treatment were: TC 420 mg/dl, LDL-c 320 mg/dL, TG 155mg/dL and HDL-c 49 mg/dL. Treatment with atorvastatin (15 mg/day) lowered her TC and LDL-c levels to 289 and 233 mg/dL respectively.

**G(-2)R mutation analysis**

This mutation ((58G>A, GGG>AGG, Gly(-2)Arg) was characterized by automatic sequencing of the 215 bp fragment from exon 1 of the LDL-r gene on analysing this fragment in patients clinically diagnosed as FH. Purified PCR product from DNA sample were directly sequenced in both directions using the amplification primers Ex1F (SEQ ID NO:2) and Ex1R (SEQ ID NO:3) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) according to the protocol described by the manufacturer. Sequences were detected using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc. Fullerton), and analyzed with CEQ 8000 software. The 58G>A change was confirmed by sequencing a second PCR product. Alternatively, this mutation could be analyzed with the microarray ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 220, SEQ ID NO: 221, SEQ ID NO: 222 and SEQ ID NO: 223.

G(-2)R mutation was identified in a 34 years old woman with arcurs corneae whose mother had hypercholesterolemia with a TC of 400 mg/dL. Her score for FH clinical diagnostic was 10 points following MedPed criteria. Her plasma lipid levels before treatment were: TC 354 mg/dL, LDL-c 264 mg/dL, normal TG and HDL-c of 64 mg/dL.

**EXAMPLE 2: Identification of mutations located in exon 2 of the LDLr gene.**

A 183 bp fragment of exon 2 was amplified by polymerase chain reaction (PCR) using the following desoxynucleotides: Ex2F (SEQ ID NO: 4) and Ex2R (SEQ ID NO: 5).

The amplification reaction was performed in a 50 µL final volume with 500 mg DNA in a mixture of 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 µM each dNTP, 0.2 µM each desoxyoligonucleotide and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycles were: 10 min of denaturation at 96°C, followed by 35 cycles: denaturation at 94°C for 1 min, hybridization at 59°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP) and those fragments that showed an abnormal SSCP pattern were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of a mutation identified by sequencing was subsequently analyzed by restriction analysis and with the device described "biochip".

**108delC mutation analysis**

This mutation creates a new MnlI digestion site. Fifteen microliters of the exon 1 amplified material were hidrolized with 15 units of MnlI in a total volume of 30 µL according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 150 and 33 bp in normal alleles and 118, 33 and 32 bp in mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide gel and were visualised by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device decribed ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43

108delC mutation was detected in a 50 years old woman, without any clinical skin manifestation of her hypercholesterolemia. She was diagnosed clinically as having FH MedPed score of 9 points. Premature cardiovascular disease was detected in one first

degree familial. Fasting plasma lipid levels while off hypolipidemic drug therapy were: TC (381 mg/dL), TG (142 mg/dL), LDLc (321) mg/dL) and HDLc (32 mg/dL).

**T41M mutation analysis**

This mutation (185C>T, ACG>ATG, Thr41Met) destroys a cleavage restriction site for the enzyme TaiI. Fifteen microliters of the exon 1 amplified material were hydrolized with 15 units of TaiI in a total volume of 30 µL according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length of 154 and 29 bp for normal alleles and 183 bp for mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide gel and were visualised by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 140, SEQ ID NO: 141, SEQ ID NO: 142 and SEQ ID NO: 143.

T41M mutation was detected in a 69 years old man who suffered a myocardial infartion at the age of 55 years and that has been diagnosed as having familial hypercholesterolemia with a diagnostic score of 6 points according to MedPed criteria. Evidence of premature cardiovascular event was detected in relatives. Analysis of his fasting serum without the use of lipid lowering drugs were: TC (274 mg/dL) and LDL-c (217 mg/dL) with normal TG and HDLc levels.

**C42Y mutation analysis**

This mutation (C42Y (188G>A, TGC>TAG, Cys42Tyr) was characterized by sequencing of the 183 bp fragment corresponding to exon 2 during screening for mutations in the LDL-r gene in subjects clinically diagnosed as FH. Purified PCR product from DNA sample were directly sequenced in both directions using the amplification primers Ex2F (SEQ ID NO:4) and Ex2R (SEQ ID NO:5) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) according to the protocol described by the manufacturer. Sequences were detected using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc. Fullerton), and analyzed with CEQ 8000 software. The G>A change was confirmed by sequencing a second

independent PCR product. Alternatively, this mutation could be analyzed with the microarray ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 248, SEQ ID NO: 249, SEQ ID NO: 250 and SEQ ID NO: 251

C42Y mutation was detected in a 17 years old man with arcus lipoides corneae that has been diagnosed as having familial hypercholesterolemia with a diagnostic score of 10 points, according to MedPed. His mother had severe hypercholesterolemia. Analysis of his fasting serum without the use of lipid lowering drugs were: TC (350 mg/dL) with normal TG and HDLc levels. Hypolypemiant treatment with simvastatin (20mg/day) lowered his TC and LDL-c levels to 274 and 214 mg/dL respectively.

**C74Y mutation analysis**

This mutation C74Y (284 G>A, TGC>TAC, Cys74Tyr) was identified by DNA sequencing of the 196 bp fragment from exon 3 during screening for mutations in the LDL-r gene in subjects clinically diagnosed as FH. Purified PCR product from DNA sample were directly sequenced in both directions using the amplification primers Ex3F (SEQ ID NO:6) y Ex3R (SEQ ID NO:7) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) according to the protocol described by the manufacturer. Sequences were detected using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc. Fullerton), and analyzed with CEQ 8000 software. The G>A change was confirmed by sequencing a second independent PCR product. Alternatively, this mutation could be analyzed with the microarray ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 212, SEQ ID NO: 213, SEQ ID NO: 214 y SEQ ID NO: 215.

C74Y mutation was detected in a 52 years old man with arcus cornealis, tendon xanthomas and family history of hypercholesterolemia. He has been diagnosed as having familial hypercholesterolemia with a diagnostic score of 17 points according to the MedPed criteria. Analysis of her fasting serum before the use of lipid lowering drugs were: TC (420 mg/dL) TG (96mg/dL) and HDLc (69mg/dL). Treatment with an HMGCoA reductase inhibitor (10mg/day) lowered his LDL-c levels by 22%.

**EXAMPLE 3: Identification of mutations located in exon 3 of the LDLr gene.**

A 196 bp fragment of exon 3 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex3F (SEQ ID NO: 6) y Ex3R (SEQ ID NO: 7).

DNA (500ng) was amplified in a 50 μL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 μM each dNTP, 0.2 μM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP) and those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**191-2delAinsCT mutation analysis**

As this mutation does not change the restriction map, we designed a pair of mutagenic primers to introduce the recognition site of BfaI in presence of the normal allele but not in presence of mutant allele.

A 184 bp fragment of exon 3 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex3R (SEQ ID NO: 7) y Mut191-2F (SEQ ID NO: 8).

DNA (500ng) was amplified in a 50 μL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 μM each dNTP, 0.2 μM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 72°C for 2 min, and a final

extension of 72°C for 10 min.

Fifteen microliters of PCR sample were hydrolized with 15 units of BfaI in a total volume of 30 μL according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length of 23 and 161 bp for normal alleles and 185 bp for mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed with the microarray ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 and SEQ ID NO: 47.

191-2delAinsCT mutation was detected in two unrelated families with autosomal dominant hypercholesterolemia. The index case of one of these families, was a 58 years old woman, with tendon xanthomas, xanthelasmas, angina pectoris, family history of coronary heart disease and hypercholesterolemia. She has been diagnosed as having familial hypercholesterolemia with a MedPed diagnostic score of 15 points. Her plasma lipid levels were: TC (559 mg/dL) and LDLc (467 mg/dL), TG (175 mg/dL) and HDLc (57 mg/dL). Treatment with simvastatin (40mg/day) lowered her TC and LDL-c levels to 302 and 228 mg/dL respectively.

**N59K mutation analysis**

This mutation (240C>A, AAC>AAA, Asn59Lys) destroys a cleavage endonuclease site for the enzyme HincII. Fifteen μL of PCR sample were digested with 15 units of HincII in a total volume of 30 μL according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 111 and 85 bp (normal alleles) or 196 bp (mutant alleles). These fragments were separated by electrophoresis in 8% polyacrilamide (PAA) gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed with the microarray ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50 y SEQ ID NO: 51.

N59K mutation was detected in a 43 years old man diagnosed clinically as having FH with a MedPed diagnostic score of 12 points. His plasma lipid levels without lipid lowering therapy were TC (465 mg/dL), LDLc (397 mg/dL), TG (100 mg/dL) and HDLc (48 mg/dL). The hypolypemiant treatment with simvastatin (40mg/dy) lowered his TC and LDL-c levels to 350 and 282 mg/dL respectively. On the other hand, his mother had suffered an angine pectoris at the age of 58 and he has a son of 8 years old with hypercholesterolemia TC (325 mg/dL) and LDLc (241 mg/dL).

**231delC mutation analysis**

This mutation destroys a endonuclease HaeIII digestion site. Fifteen microliters of PCR sample were digested with 15 units of HaeIII in a total volume of 30 μL according to the protocol described by the manufacturer (Gibco BRL, Carlsbad, CA, USA). The fragments obtained had a length of 76, 51, 42 and 25 bp for normal alleles and 117,51, and 27 bp for mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide (PAA) gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54 y SEQ ID NO: 55

The mutation was detected in a 37 years old woman, with arcus corneae. She was diagnosed clinically as having FH with a score of 16 points following the WHO MedPed criteria. Plasma lipid levels without lipid lowering therapy were: TC (543 mg/dL), LDLc (456 mg/dL), TG (178 mg/dL) and HDL-c (51 mg/dL). The hypolypemiant treatment with atorvastatin (40mg/day) and colestipol (20g/day) lowered her TC and LDL-c levels to 260 and 190 mg/dL respectively. Her brother suffered a myocardial infarction at the age of 38 and her son of 12 years old have hypercholesterolemia with TC concentration of 305 mg/dL.

**313+1insT mutation analysis**

This mutation creates a new cleavage site for the restriction endonuclease TruII . Fifteen microliters of the exon 3 amplified material were hydrolized with 15 units of TruII in a total volume of 30 μL according to the protocol described by the manufacturer

(Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 196 bp for normal alleles and 162 and 34 bp for mutant alleles. These fragments were separated by electrophoresis in 3% NuSieve agarose gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58 y SEQ ID NO: 59.

313+1insT mutation was detected in a 53 years old woman, with xanthomas and arcus corneae. No premature cardiovascular events was detected in her available family members. She was diagnosed clinically as having FH with a MedPed score of 19 points. Analysis of her fasting serum lipid levels without the use of lipid lowering drugs were: TC (574 mg/dL) and LDLc (505 mg/dL) with normal TG and HDLc levels. After lipid lowering hypolypemiant treatment with simvastatin (80 mg/day) and colestipol (20 g/day) their TC and LDL-c levels decreased at 282 mg/dL and 225 mg/dL respectively.

**EXAMPLE 4: Identification of mutations located in exon 4 of the LDLr gene.**

A 242 bp fragment of LDL-r gene from the 5´ region of exon 4 (exon 4A) was amplified by polymerase chain reaction (PCR) using the following primers: Ex 4AF (SEQ ID NO: 9) y Ex 4AR (SEQ ID NO: 10

DNA (500ng) was amplified in a 50 μL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 μM each dNTP, 0.2 μM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycles were: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**338del16 mutation analysis**

This mutation creates a new cleavage site for the restriction endonuclease Van91I. Fifteen microliters of the exon 4 amplified material were hydrolized with 15 units of Van91I in a total volume of 30 µL according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 242 bp for normal alleles and 174 and 52 bp for mutant alleles. These fragments were separated by electrophoresis in 2% agarose gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides SEQ ID NO: 144, SEQ ID NO: 145, SEQ ID NO: 146 y SEQ ID NO: 147.

338del16 mutation was detected in three unrelated families with autosomal dominant hypercholesterolemia. One index case of these families, was a 40 years old man with xanthomas and arcus corneae, TC 542 mg/dL and LDLc 441 mg/dL and normal TG and HDLc levels. He was diagnosed as having FH with a MedPed score of 19 points. No cardiovascular event was detected in his available family members. The hypolypemiant treatment with atorvastatin (10mg/day) lowered his plasma TC and LDL-c levels to 293 and 218 mg/dL respectively.

**5090insC mutation analysis**

As this mutation does not change the restriction map, we designed a pair of mutagenic primers to introduce one recognition site for the restriction enzyme MnlI in presence of the mutant allele but not in presence of normal allele.

A 244 bp fragment of exon 4A was amplified by polymerase chain reaction (PCR) using the following primers: Ex4AF (SEQ ID NO: 9 and Mut509insCR (SEQ ID NO: 11).

DNA (500ng) was amplified in a 50 µL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 µM each dNTP, 0.2 µM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycles were: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at

94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

Fifteen microliters of PCR sample were digested with 15 units of MnlI in a total volume of 30 μL according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 141, 99 and 4 bp for normal alleles for 141, 88, 12 and 4 bp in mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed by the device described ("biochip") using in the slide the oligonucleotides SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62 and SEQ ID NO: 63.

509insC mutation was detected in a 44 years old woman with hypercholesterolomia TC (477 mg/dL) and LDLc (394 mg/dL) with normal without personal and familial history of premature coronary heart disease. Their diagnostic score was 9, following the MedPed criteria. She has two brothers with hypercholesterolemia at a c-LDL concentration beyond 95.

**451del3 mutation analysis**

This mutation (451del3) was characterized by DNA sequencing of the 242 bp fragment from exon 4 (4A) during screening for mutations in the LDL-r gene in subjects clinically diagnosed as having FH. Purified PCR product from DNA sample were directly sequenced in both directions using the amplification primers Ex4AF (SEQ ID NO:9) and Ex 4AR (SEQ ID NO:10) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) according to the protocol described by the manufacturer. Sequences were detected using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc. Fullerton), and analyzed with CEQ 8000 software. The three base pair deletion was confirmed by sequencing a second PCR products. Alternatively, this mutation could be analyzed with the microarray ("biochip") by using in the slide the oligonucleotides: ID NO: 172, SEQ ID NO: 173, SEQ ID NO: 174 and SEQ ID NO: 175.

451del3 mutation was detected in a 36 years old man with arcus lipoides corneae that has been previously suffered a myocardial infartion at age of 34. He has two children 2 and 8 years olds with TC of 320 and 275 mg/dl respectively. He was diagnosed clinically as having FH with a score of 17 points. Analysis of his fasting serum lipids without the use of lipid lowering drugs were TC 449 mg/dl, LDL-c 367 mg/dL, TG 218 mg/dL and c-HDL-c 38 mg/dL. Treatment with simvastatin (40mg/day) lowered his LDL-c level to 270 mg/dL.

**EXAMPLE 5: Identification of mutations located in exon 4B of the LDLr gene.**

A 237 bp fragment of 3′ exon 4 (exon 4B) of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex4BF (SEQ ID NO: 12) and Ex4BR (SEQ ID NO: 13).

DNA (500ng) was amplified in a 50 µL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 µM each dNTP, 0.2 µM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycles were: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the microarray ("biochip").

**D157G mutation analysis**

This mutation (533A>G, GAT>GGT, Asp195Gly) creates a new digestion site for the endonuclease HphI. Fifteen microliters of the exon 4B amplified material were hydrolized with 15 units of HphI in a total volume of 30 µL according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length of 237 bp for normal alleles and 175 and 62 bp for mutant alleles. These fragments

were separated by electrophoresis in 3% NuSieve agarose gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66 and SEQ ID NO: 67.

D157G mutation was detected in a 32 years old woman with hypercholesterolemia. No cardiovascular event was detected in her family. She was diagnosed clinically as having possible FH with a MedPed score of 6 points. Analysis of her fasting serum lipids before the use of lipid lowering therapy were: TC (358 mg/dL) and LDLc (296 mg/dL) with normal TG and HDLc levels. Treatment with atorvastatin (10mg/day) lowered her plasma TC and LDL-c levels to 212 and 140mg/dL respectively. Her father also had elevated levels of plasma cholesterol TC 364 mg/dL, as well as her grandmother 341 mg/dL.

**C195R mutation analysis**

This mutation (646T>C, TGT>CGT, Cys195Arg) creates a BshNI digestion site. Fifteen microliters of the exon 4B amplified material were hydrolized with 15 units of BshNI in a total volume of 30 µL according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 237 bp, corresponding to the amplified material without hydrolizing, for normal alleles and 159 and 78 bp for mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide (PAA) gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed by the device described ("biochip") using in the slide the oligonucleotides SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70 and SEQ ID NO: 71.

C195R mutation was detected in a 64 years old woman who have hypercholesterolemia and arcus corneae. Premature cardiovascular disease was detected in her mother. She was diagnosed clinically as having FH with a MedPed score of 11 points Plasma lipid levels without lipid lowering therapy were: TC (560 mg/dL) and LDLc (468 mg/dL) with normal TG and HDLc levels.

**675del15 mutation analysis**

This mutation was identified by heteroduplexes analysis; the electrophoresis in 8% polyacrilamide gel of the exon 4B amplified material PCR visualized by staining with ethidium bromide, showed the presence of heteroduplexes bands instead of the corresponding normal and mutated homoduplexes. The fragments obtained had a length of 237 bp in normal alleles and 222 bp in mutant alleles. The heteroduplex band migrated more slowly because of the formation of the bubble between the michtmached sequences. Alternatively, this mutation could be analyzed with the microarray ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74 y SEQ ID NO: 75.

675del15 mutation was detected in a 63 years old woman, clinically diagnosed as having FH with a MedPed score of 8 points. No cardiovascular event was detected in her family. An untreatment lipid determination gave us the following results: TC (450 mg/dL) and LDLc (379 mg/dL) with normal TG and HDLc levels. No family members were available to complete the genetic study.

**684dup12 mutation analysis**

This mutation was analysed by digestion of the exon 4B amplified fragment with MnlI enclonuclease restriction site. The addition of 12 bp produced by the mutation, allows detecting the presence of the mutation in the exon 4B amplified material by electrophoresis in 8% polyacrilamide gel and tinction of the gel with ethidium bromide. Additionally, fifteen microliters of the exon 4B amplified material were hydrolized with 15 units of MnlI in a total volume of 30 μL according to the protocol described by the manufacturer (Fermentas, Inc., Hanover, MD, USA). The fragments obtained had a length of 192 and 45 bp for normal alleles and 204 and 45 bp for mutant alleles. These fragments were separated by electrophoresis in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78 and SEQ ID NO: 79

684dup12 mutation was detected in two unrelated families having autosomal dominant hyperclolesterolemia. The index case of one of these families, was a 63 years

old man, with xanthomas and arcus corneae He has been suffered a myocardial infarction at the age of 55 and was diagnosed clinically as having FH with a MedPed score of 17 points. No cardiovascular event was detected in his family. Plasma lipid levels without lipid lowering therapy were: TC (469 mg/dL), LDLc (408 mg/dL),TG (100 mg/dL) and HDLc 41 mg/dL.

**D200V mutation analysis**

This mutation (D200V (662A>T, GAC>GTC, Asp200Val)) was identified by DNA sequencing of the 237 bp fragment from exon 4 (4B) during screening for mutations in the LDL-r gene in subjects clinically diagnosed as having FH. Purified PCR product from DNA sample were directly sequenced in both directions using the amplification primers Ex4BF (SEQ ID NO:12) and Ex 4BR (SEQ ID NO:13) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) according to the protocol described by the manufacturer. Sequences were detected using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc. Fullerton), and analyzed with CEQ 8000 software. The 662A>T change was confirmed by sequencing a second PCR product. Alternatively, this mutation could be analyzed with the device described ("biochip") using in the slide the oligonucleotides: SEQ ID NO: 232, SEQ ID NO: 233, SEQ ID NO: 234 and SEQ ID NO: 235.

D200V mutation was detected in a family with autosomal dominant hypercolesterolemia. The subject was a 43 years old woman with family history of hypercholesterolemia in infancy and whose mother and broter presented LDL-c levels above the 95 percentile. She was diagnosed clinically as having familial hypercholesterolemia with a score of 8 points, following the MedPed criteria. Analysis of her fasting serum lipids using lipid lowering drug pravastatin (40 mg/day) were TC 329 mg/dl, LDL-c 273 mg/dL, TG 73 mg/dL and HDL-c 41 mg/dL.

**S205Cmutation analysis**

This mutation S205C (677C>G, TCT>TGT, Ser205Cys) was identified by DNA sequencing of the 237 bp fragment from exon 4 (4B) during screening for mutations in the LDL-r gene in subjects clinically diagnosed as having FH. Purified PCR product from

DNA sample were directly sequenced in both directions using the amplification primers Ex4BF (SEQ ID NO:12) and Ex 4BR (SEQ ID NO:13) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The C>G change observed was confirmed by automatically sequencing a second PCR product of the same ample. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 228, SEQ ID NO: 229, SEQ ID NO: 230 and SEQ ID NO: 231.

S205C mutation was detected in a 39 years old woman with family history of hypercholesterolemia (mother and brother TC 450 mg/dL and 500 mg/dL respectively) with two children with TC above the 95 percentile. She was diagnosed clinically at 20 years old as having familial hypercholesterolemia with a MedPed score of 8 points The plasmatic lipid concentrations prior to pharmalogical treatment were: TC 390 mg/dl, LDL-c 325 mg/dL and HDL-c 35 mg/dL. Treatment with simvastatin (10mg/day) lowered her plasma LDL-c level to 270 mg/dL.

**EXAMPLE 6: Identification of mutations located in exon 6 of the LDLr gene.**

A 179 bp fragment of exon 6 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex6F (SEQ ID NO: 14) y Ex6R (SEQ ID NO: 15).

DNA (500ng) was amplified in a 50 □L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 □M each dNTP, 0.2 □M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**C255G mutation analysis**

As this mutation C255G (826T>G, TGC>GGC, Cys255Gly) does not change the restriction map, a desoxyoligonucleotide was designed and synthetized with nonadjoining base to introduce the recognition site of BstUI restriction enzyme in presence of the mutuant allele, which disappears in the presence of normal allele.

A 163 bp fragment of exon 6 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex6R (SEQ ID NO: 15) and MutC255GF (SEQ ID NO: 16).

DNA (500ng) was amplified in a 50 □L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 □M each dNTP, 0.2 □M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

Fifteen $\mu$L of PCR sample were digested with 15 units of BstUI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length of 163 bp for normal alleles and 141 and 22 bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed wit the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82 and SEQ ID NO: 83.

C255G mutation was detected in a 63 years old woman, with family history of hypercholesterolemia. A lipid determination with treatment were: TC (439 mg/dL) and LDLc (355 mg/dL) with normal TG and HDLc levels. She was diagnosed clinically as having familial hypercholesterolemia with a MedPed score of 8 points

**E291X mutation analysis**

As this mutation E291X (934G>T, GAG>TAG, Asp291Stop) does not change the restriction map, a desoxyoligonucleotide was designed and synthesized with a nonadjoining base to create a recognition site fro the restriction enzyme SspI in presence of the mutated allele which disappears in the presence of the normal allele.

A 164 bp fragment of exon 6 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex6F (SEQ ID NO: 13) and Mut E291XR (SEQ ID NO: 17).

DNA (500ng) was amplified in a 50 □L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 □M each dNTP, 0.2 □M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

Fifteen μL of PCR sample were digested with 15 units of SspI in a total volume of 30 μL according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 164 bp (non-digested fragment) for normal alleles and 144 and 20 bp for mutant alleles. These fragments were electrophoresed in 3% NuSieve agarose gel and were visualized by staining with ethidium bromide.

Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86 y SEQ ID NO: 87.

E291X mutation was detected in a family with autosomal dominant hypercholesterolemia family. The subject was a 44 years old man with arcus corneae and concentrations: TC (381 mg/dL), HDLc (45 mg/dL), TG (111 mg/dL) and LDLc (314 mg/dL). His clinical diagnosis of hypercholesterolemia familiar reached a score of 12 points, according to the MedPed criteria. Combined lipid lowering treatment with simvastatin (40mg/day) and colestiramin (12g/day) lowered his plasma TC and LDL-c levels to 253 mg/dL and 188 mg/dL.

**818del8 mutation analysis**

This mutation was identified by heteroduplexe analysis; the electrophoresis in 8% polyacrilamide (PAA) gel of amplified product of exon 6 visualized by staining with ethidium bromide, showed the presence of heteroduplexes bands instead of the corresponding normal and mutated homoduplexes of 179 and 171 bp, readily distinguishable in the gel after staining with ethidium bromide. The two heteroduplex bands migrated more slowly because of the formation of the bubbles between the mismatched sequences.

In addition the mutation could be confirmed by PCR amplification of exon 6 and restriction analysis, the 818del8 mutation creates a new MaeIII endonuclease restriction site . Fifteen μL of PCR sample were digested with 15 units of MaeIII in a total volume of 30 μL according to the protocol described by the manufacturer (Roche Diagnostics,

Manheim, Germany). The fragments obtained had a length of 118, 34 and 27 bp for normal alleles and 118 and 53 bp for mutant alleles. These fragments were electrophoresed in 8% of polyacrilamide and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO:162 and SEQ ID NO: 163.

818del8 mutation was detected in a 69 years old woman, clinically diagnosed as having FH with a MedPed score of 10 points. Her two sons have hipercholesterolemia with plasma TC levels of 382 and 304 mg/dL respectively. The clinical diagnosis of family hypercholesterolemia reached a score of 10 points on MedPed criteria. The plasmatic lipid concentrations prior to pharmacological treatment were: TC (530 mg/dL) and LDLc (439 mg/dL) TG( 170 mg/dL and HDLc 57 mg/dL Lipid lowering treatment with cerivastatin (0.4mg/day) reduced her LDL-c to 363 mg/dL.

**R279G mutation análisis**

This mutation R279G (898A>G, AGA>GGA, Arg279Gly) was identified by automatic sequencing of the 179 bp fragment from exon 6 during screening for mutations in the LDL-r gene in subjects clinically diagnosed as FH. Purified PCR product from DNA sample were directly sequenced in both directions using the amplification primers Ex6F (SEQ ID NO:14) and Ex6R (SEQ ID NO:15) and the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) The fragments generated by the sequence reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The A>G change observed was confirmed by sequencing a second PCR product of the same sample.

Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202 and SEQ ID NO: 203.

R279G mutation was identified in a 59 years old woman with xantelasmas and family history of hypercholesterolemia in the father and two brothers. The score for FH clinical diagnostic was 10 points Plasma lipid levels before treatment were: TC 384 mg/dL, LDL-c 314 mg/dL and normal TG and HDL-c levels. Lipid lowering treatment with simvastatin (80 mg/day) lowered her LDL-c to 167 mg/dL.

**EXAMPLE 7: Identification of mutations located in exon 7 of the LDLr gene.**

A 234 bp fragment of exon 7 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers Ex7F (SEQ ID NO: 18) y Ex7R (SEQ ID NO: 19.

DNA (500ng) was amplified in a 50 □L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 □M each dNTP, 0.2 □M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of a mutation identified by secuencing was subsequently analysed by restriction analysis and with the device described previously ("biochip").

**941-39C>T mutation analysis**

This mutation destroys an ApaI digestion site. Fifteen $\mu$L of exon 7 PCR sample were digested with 15 units of ApaI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD,USA). The fragments obtained had a length of 186, 26 and 22 bp for normal alleles and 208 and 26 bp for

mutant alleles. These fragments were electrophoresed in 8% polyacrilamide (PAA) gel and were visualised by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90 y SEQ ID NO: 91.

941-39C>T mutation was detected in four unrelated families who had the characteristic in common of having an autosomal dominant familiar hypercholesterolemia. The index case of one of these families was a 61 years old woman who had suffered a myocardium attack and with a family history of premature cardiovascular disease. She was clinically diagnosed as having FH with a MedPed score of 7 points. Plasma lipid levels before treatment were: TC (340 mg/dL) and LDLc (248 mg/dL) with TG 136 mg/dL and HDL-c 65 mg/dL. After lipid lowering treatment with atorvastatin (20 mg/day) TC and LDL-c levels decreased at 233 mg/dL and 144 mg/dL respectively with no significant changes in TG and HDL-c levels.

**C319Y mutation analysis**

This mutation C319Y (1019G>A, TGC>TAC, Cys319Tyr) creates a new RsaI endonuclease digestion site. Fifteen $\mu$L of PCR sample were digested with 15 units of RsaI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Gibco BRL, Carlsbad, CA, USA). The fragments obtained had a length of 234 bp (fragment without digestion) in normal alleles and 136 and 98 bp in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide (PAA) gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94 y SEQ ID NO: 95

C319Y mutation was detected in a family with autosomal dominant familial hypocholesterolemia. The subject was a 43 years old man, with arcus corneae and xanthomas at Achiles tendon and dorsum of the hands and corneal arc and with a 17 years old son with total plasmatic cholesterol of 384 mg/dL. . His father had suffered sudden death at 45 years of age. He was clinically diagnosed as having FH with a

MedPed score of 22 points. Plasma lipid levels before treatment were: TC (428 mg/dL) and LDLc (372 mg/dL) with normal TG level.

**1054del11 mutation analysis**

This mutation destroys an endonuclease restriction site for the HphI enzyme. Fifteen $\mu$L of the amplified material of exon 7 were digested with 15 units of HphI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Gibco BRRL, Carlsbad, CA, USA). The fragments obtained had a length of 189 and 45 bp in normal alleles and 223 bp in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98 y SEQ ID NO: 99

1054del11 mutation was detected in a family with autosomal dominant familial hypercholesterolemia. The subject wa a 43 years old man with xanthomas at Achiles tendon and premature cardiovascular disease, with a first degree relative who suffered a premature myocardial infartion. He was clinically diagnosed as having FH with a MedPed score of 16 points . Plasma lipid levels before treatment were: TC (480 mg/dL), LDLc (416 mg/dL), TG (95 mg/dL and HDLc 36 mg/dL.

**EXAMPLE 8: Identification of mutations located in exon 8 of the LDLr gene.**

A 220 bp fragment of exon 8 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex8F (SEQ ID NO:148) and Ex8R (SEQ ID NO: 149)..

DNA (500ng) was amplified in a 50 □L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification

cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

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**1186+5 G>A mutation analysis**

This mutation (1186+5 G>A) was characterized by automatic sequencing of the 220 bp fragment from exon 8 during screening for mutations in the LDL-r gene in subjects clinically diagnosed as FH. The sequencing reaction was performed in a PE Gene Amp System 9700 thermocyclator using the reagents of the kit CEQ 2000 Dye Terminator Cycle Sequencing with Beckman Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex8BF (SEQ ID NO:148) and Ex8BR (SEQ ID NO:149).

The fragments generated by the sequencing reaction were analysed in a automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The G>A change was confirmed by sequencing a second PCR product of the same sample. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 188, SEQ ID NO: 189, SEQ ID NO: 190 y SEQ ID NO: 191.

This mutation was identified in two unrelated families with autosomal dominant hipecholesterolemia. The index case of one of these families was a 34 years old woman with xantelasmas, arcus corneae, tendon xanthomata and family history of hypercholesterolemia. . She was clinically diagnosed as having FH with a MedPed score of 21 points. Plasma lipid levels before treatment were: TC 411 mg/dL, LDL-c 346 mg/dL and normal TG and HDL-c levels. Lipid lowering treatment with cerivastatin (0.2 mg/day) reduced her LDL-c to 222 mg/dL.

**EXAMPLE 9: Identification of mutations located in exon 9 of the LDLr gene.**

A 224 bp fragment of exon 9 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex9F (SEQ ID NO: 20) and Ex9R (SEQ ID NO: 21).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M of each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycles were: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**1197del9 mutation analysis**

This mutation was analyzed by heteroduplex analysis; the electrophoresis in 8% polyacrilamide gel of PCR products visualized by staining with ethidium bromide, showed the presence of heteroduplexes bands instead of the corresponding normal and mutated homoduplexes. The fragments obtained had a length of 224 bp for normal alleles and 215 bp for mutant alleles. The heteroduplex band migrated more slowly because of the formation of the bubble between the michtmached sequences. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102 y SEQ ID NO: 103.

1197del9 mutation was detected in eight unrelated families having the characteristic in common of having an autosomal dominant familial hypercholestrolemia. Index case of one of these families was a 45 years old woman, with xanthomata who suffered an angine pectoris at the age of 41. Her father suffered a myocardial infarction at the age of 36. She was clinically diagnosed as having FH with a MedPed score of 18 points. Plasma lipid levels before treatment were: TC (525 mg/dL), LDLc (443 mg/dL), TG (163 mg/dL) and HDLc (49 mg/dL). Lipid lowering treatment with atorvastatin (20 mg/day) reduced her LDL-c to 323 mg/dL.

**Y379X mutation analysis**

This mutation Y379X (1200C>A, TAC>TAA, Tyr379Stop) destroy a cleavage site for the restriction endonuclease MnlI. Fifteen $\mu$L of PCR sample were digested with 15 units of MnlI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Gibco BRL, Carlbad,CA.USA). The fragments obtained had a length of 87, 56, 34, 22, 18, 4, and 3 bp for normal alleles and 87, 56, 38, 22, 18, and 3 bp for mutant alleles. These fragments were electrophoresed in 16% polyacrilamide (PAA) gel and in this way it was possible to distinguish the 34 and 38 bp bands which differentiate both alleles by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the

oligonucleotides SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106 y SEQ ID NO: 107.

Y379X mutation was detected in a family with autosomal dominant hypercholestrolemia. The subject from such family was a 69 years old man. His father had died of a myocardial infartion at age 50 and had two children with total plasmatic cholesterol above the 95 percentile. He was clinically diagnosed as having FH with a MedPed score of 7 points. A lipid determination without treatmentgave us the following results: TC (381 mg/dL) and LDLc (306 mg/dL) with normal TG and HDLc levels. Lipid lowering treatment with atorvastatin (20 mg/day) reduced his LDL-c to 259 mg/dL

**1207delT mutation analysis**

This mutation destroys a cleavage site for the restriction enzyme MboII. Fifteen $\mu$L of amplified material of exon 9 were digested with 15 units of MboII in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 140, 46, 35, and 3 bp for normal alleles and 140, 48, and 35 bp for mutant alleles. These fragments were electrophoresed in 16% polyacrilamide gel and by staining with ethidium bromide the 46 and 48 bp bands could be distinguished, which differentiate both alleles. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110 y SEQ ID NO: 111.

1207delT mutation was detected in a member of family with autosomal dominant hypercholesterolemia. The subject was a 35 years old woman. The MedPed score for FH clinical diagnostic was 9 points. The Plasma lipid levels without lipid lowering treatment were: TC (429 mg/dL), LDLc (345 mg/dL), TG (188 mg/dL) and HDLc (46 mg/dL). Combined lipid lowering treatment with simvastatin (40 mg/day) and colestipol (5g/day) reduced her TC and LDL-c to 220 mg/dL and 137 mg/dL without significant changes in her TG and HDL-c levels.

**Y421X mutation analysis**

This mutation Y421X (1326C>G, TAC>TAG, Tyr421Stop) creates a new cleavage site for the endonuclease BfaI. Fifteen μL of PCR sample were digested with 15 units of BfaI in a total volume of 30 μL according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length of 224 bp (fragment without digestion) for normal alleles and 164 and 60 bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114 y SEQ ID NO: 115.

Y421X mutation was detected in three unrelated families having in common autosomal deominant familial hipercholesterolemia. The index case of one of these families was a 71 years old woman, with arcus corneae, tendon xanthomas and xantelasmas. Her father had suffered a myocardial infarction at the age of 51 and had a son with marked hypercholesterolemia (TC 367 mg/dL). She was clinically diagnosed as having FH with a MedPed score of 16 points. The plasmatic concentrations of lipids without the use of lipid lowering drugs were: TC (615 mg/dL) and LDLc (550 mg/dL) with normal TG and HDLc levels.

**1204insT mutation analysis**

This mutation destroys a cleavage site for the endonuclease MboII. Fifteen μL of exon 9 PCR sample were digested with 15 units of MboII in a total volume of 30 μL according to the protocol described by the manufacturer (Amersham Pharmacia, NJ, USA). The fragments obtained had a length of 141, 45, 35 and 3 bp in normal alleles and 141, 45 and 39 pb in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170 y SEQ ID NO: 171

This mutation was detected in a girl 12 years old. Her father and 7 years old brother had hypercholesterolemia with TC levels of 412 and 321 mg/dL respectively. The MedPed score for FH clinical diagnostic was 9 points. Analysis of her fasting serum lipid levels without the use of lipid lowering drugs were TC 332 mg/dL, LDL-c 267 mg/dL with normal TG and HDL-c levels. Lipid lowering treatment with resins (15 g/day) reduced the LDL-c levels to 248 mg/dL.

**EXAMPLE 10: Identification of mutations located in exon 10 of the LDLr gene.**

A 278 bp fragment of exon 10 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex10F (SEQ ID NO: 22) and Ex10R (SEQ ID NO: 23

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 74°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**1432delG mutation analysis**

As this mutation does not change the restriction map, a mismatched desoxyoligonucleotide was designed and synthetized to introduce the recognition site for

the NaeI restriction enzyme in presence of the mutant allele that disappears in the presence of normal allele.

A 200 bp fragment of exon 10 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex10R (SEQ ID NO: 23) and Mut1432delGF (SEQ ID NO: 24).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

Fifteen $\mu$L of PCR sample were digested with 15 units of NaeI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 200 bp (undigested fragment) for normal alleles and 179 and 20 bp in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118and SEQ ID NO: 119.

1432delG mutation was detected in a family with autosomal dominant hypercholesterolemia. The subject was a 53 years old woman with tendon xanthomas who had suffered a myocardial infartion, moreover with a family history of premature cardiovascular disease. She was clinically diagnosed as having FH with a MedPed score of 15 points. An lipid analysis without use lipid lowering therapy gave us the following results: TC (548 mg/dL) and LDLc (470 mg/dL) with normal TG and HDLc levels.

**T433N mutation analysis**

This mutation T433N (1361C>A, ACC>AAC, Tyr433Asn) was characterized by automatic sequencing of the 278 bp fragment from exon 10 of the LDL-r gene on analysing this fragment in subjects clinically diagnosed as having FH. The sequencing reaction was developed in the thermocycler PE Gene Amp System 9700 using the reagents of the kit CEQ 2000 Dye Terminator Cycle Sequencing with Beckman Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex10F (SEQ ID NO: 22) and Ex10R (SEQ ID NO: 23). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The C>A change was confirmed by sequencing a second PCR product from the same sample. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158 and SEQ ID NO: 159.

T433N mutation was detected in a 50 years old man with arcus corneae and family history of autosomal dominant hypercholesterolemia and daughter 21 years old with TC levels of 310 mg/dL. The MedPed score for FH clinical diagnostic was 6 points. Analysis of his plasmatic lipip concentrations beforfe beginning pharmalogical treatment were TC 318mg/dl, LDL-c 249 mg/dL with normal TG and HDL-c. Lipid lowering therapy with lovastatin ( 20mg/day) reduced his LDL-c to 199 mg/dL

**<u>Análisis of the mutation T446I</u>**

This mutation T446I (1400C>T, ACC>ATC, Tyr446Ile) was characterized by automatic sequencing of the 278 bp fragment from exon 10 of the LDL-r gene in subjects clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the reagents of the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex10F (SEQ ID NO: 22) and Ex10R (SEQ ID NO: 23). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The C>T change was confirmed by sequencing a second PCR product from the same sample. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the

oligonucleotides: SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206 and SEQ ID NO: 207.

T446I mutation was detected in a 64 years old woman with a background of premature cardiovascular disease (an angor at 62 years of age) and with two brothers with hypercholesterolemia who been suffered a myocardial infartion at 40 and 46 years of age respectively. She was diagnosed clinically as having FH with a MedPed score of 9 points. The plasmatic concentrations of lipids under pharmalogical treatment with pravastatin were: TC (352 mg/dL) and LDLc (281 mg/dL) with normal TG and HDLc levels. After lipid lowering treatment with simvastatin 20 mg/day the LDL-c levels decreased to 150 mg/dL.

**Análisis de la mutación 1423delGC/insA**

This mutation 1423delGC/insA destroys a cleavage site for the endonuclease MvaI. Fifteen $\mu$L of exon 10 PCR product were digested with 15 units of MvaI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Fermentas Inc., Henover, MD, USA). The fragments obtained had a length of 150 and 128 bp for normal alleles and 128, 87 and 63 bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166 y SEQ ID NO: 167.

This mutation was detected in a 34 years old man with parental history of hypercholesterolemia. The MedPed score for FH clinical diagnosis was 9 points.The plasmatic lipid concentrations prior to pharmalogical treatment were: Analysis of her fasting serum lipid levels without the use of lipid lowering drugs were: Total TC 554 mg/dL, LDL-c 422 mg/dL with normal TG and HDL-c levels. Lipid lowering treatment with atorvastatine (10 g/day) lowered his LDL-c levels to 406mg/dL.

**EXAMPLE 11: Identification of mutations located in exon 11 of the LDLr gene.**

A 194 bp fragment of exon 11 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex11F (SEQ ID NO: 25) y Ex11R (SEQ ID NO: 26).

DNA (500ng) was amplified in a 50 μL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 μM each dNTP, 0.2 μM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of a mutation identified by sequencing was then analysed by restriction analysis and by the device described previously "biochip".

**W515X mutation analysis**

This mutation W515X (1607G>A, TGG>TAG, Trp515Stop) creates a new BfaI digestion site. Fifteen μL of PCR sample were digested with 15 units of BfaI in a total volume of 30 μL according to the protocol described by the manufacturer (NEB , Beverly, MA, USA). The fragments obtained had a length of 164 and 30 bp for normal alleles and 97, 67 and 30 bp for mutant alleles. These fragments were electrophoresed in 3% NuSieve agarose gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122 and SEQ ID NO: 123.

W515X mutation was detected in a 39 years old man, with arcus corneae whose father had suffered a myocardial infartion at age 50. He has been diagnosed as having familial hypercholesterolemia with a MedPed diagnostic score of 13 points. The plasmatic lipid concentrations without pharmacological treatment were: TC (364 mg/dL)

and LDLc (308 mg/dL) with normal TG and HDLc levels. The subject's father, two brothers and a son had cholesterol levels above the 95 percentile.

**Análisis of the mutation [1587-5del5; 1587del31]**

This mutation [1587-5del5; 1587del31] was identified by automatic sequencing of the 194 pb fragment from exon 11 of the LDL-r gene on analysing this fragment in subjects clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers (SEQ ID NO: 25) y Ex11R (SEQ ID NO: 26).

The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. This deletion was confirmed by electrophoresis in 2% agarose gel after which bands of 194 and 258 bp could be observed corresponding to the normal allele and mutated allele respectively. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 256, SEQ ID NO: 257, SEQ ID NO: 258 and SEQ ID NO: 259.

[1587-5del5; 1587del31] mutation was detected in a 43 years man with arcus corneae and family history of autosomal dominant hypercholesterolemia (father and son with hypercholesterolemia) and evidence of cardiovascular disease in the family (his father suffered a myocardial infartion at age 50). He was diagnosed clinically as having FH with a MedPed score of 9 points. The plasmatic lipid concentrations before pharmacology treatment were: TC (345mg/dL) and TG (160 mg/dL) and HDLc(34 mg/dL). After combined lipid lowering treatment with simvastatin 40 mg/day and colestipol 10g/day the LDL-c levels decreased to 208 mg/dL.

Analysis of the g516x mutation

***G516X MUTATION ANALYSIS***

This mutation (1609G>T, GGA>TGA, Gly516Stop) creates a new HphI digestion site. Fifteen L of amplified material of exon 11 were digested with 15 units of HphI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length 139, 43 and 12 bp for normal alleles

and 81, 58, 43 y 12 p¡bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 176, SEQ ID NO: 177, SEQ ID NO: 178 and SEQ ID NO: 179

G516X mutation was detected in a 20 years old woman, with tendon xanthomas and family history of hipercholesterolemia (mother and two adolescent brothers with LDL-c levels above the 95 percentile). She has been diagnosed as having familial hypercholesterolemia with a MedPed diagnostic score of 17 points. The plasmatic lipid concentrations prior to pharmacological treatment were: TC 476 mg/dL, LDL-c 403 mg/dl and normal TG and HDL-c levels. After lipid lowering treatment with an HMGCoA reductase inhibitor the LDL-c levels decreased to 202 mg/dL

**H562Q mutation analysis**

This mutation (1749C>A, CAC>CAA, His562Gln) was identified by automatic sequencing of the 194 pb fragment from exon 11 of the LDL-r gene on analysing this fragment in patients clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers (SEQ ID NO: 25) y Ex11R (SEQ ID NO: 26). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The change observed C>A was confirmed by automatic sequencing of a second PCR product of the same sample. Alternatively, this mutation can be analysed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 208, SEQ ID NO: 209, SEQ ID NO: 210 and SEQ ID NO: 211.

H562Q mutation was detected in a 37 years woman with family history of autosomal dominant hypercholesterolemia, (father with hypercholesterolemia and suffered a myocardial infartion at age of 48 and her son at age 13 with TC level of 500 mg/dL). She was diagnosed clinically as having FH with a MedPed score of 9 points. The plasmatic lipid concentrations before pharmacological treatment were: TC

(350mg/dL) with normal TG and HDLc levels. After lipid lowering treatment with atorvastatin 20 mg/day the TC level lowered to 333 mg/dL.

**EXAMPLE 12: Identification of mutations located in exon 12 of the LDLr gene.**

A 236 bp fragment of exon 12 was amplified by polymerase chain reaction (PCR) using the following primers: Ex12F (SEQ ID NO: 150) and Ex12R (SEQ ID NO: 151).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

The PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously ("biochip").

**E579D mutation analysis**

This mutation E579D (1800G>C, GAG>GAC, Glu579Asp)) was identified by automatic sequencing of 236 bp fragment from exon 12 of the LDL-r gene on analyzing this fragment in patients clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex12F (SEQ ID NO: 150) and Ex12R (SEQ ID NO:151). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The change observed G>C was confirmed by automatic sequencing of a second PCR product of the same sample. Alternatively, this mutation can be analysed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 224, SEQ ID NO: 225, SEQ ID NO: 226 and SEQ ID NO: 227.

E579D mutation was detected in a 49 years old man with family history of autosomal dominant hypercholesterolemia (ather with TC 450mg/dL and his brother and two adolescent children with LDL-c levels 95 percentile). He was diagnosed clinically as having FH with a MedPed score of 8 points. Analysis of his plasmatic lipid concentrations prior to pharmacological treatment were: TC (320mg/dL), LDL-c (250 mg/dL) with normal TG and HDL-c levels. After lipid lowering treatment with atorvastatin (10 mg/day) the LDL-c level lowered to 187 mg/dL.

**1815del11 mutation analysis**

This mutation was able to be identified by heteroduplexes analysis. The electrophoresis in 8% polyacrilamide (PAA) gel of exon 12 PCR amplified material when mutuation exists showing the presence of heteroduplex bands of an apparent greater molecular size than the two homoduplex bands of 236 and 225 bp, readily distinguished in the gel following staining with ethidium bromide. The two bands of the heteroduplexes that form migrate at a slower speed as a result of the formation of bubbles between the mismatched sequences. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 184, SEQ ID NO: 185, SEQ ID NO: 186 y SEQ ID NO: 187.

1815del11 mutation was identified in four unrelated families with autosomal dominant familial hypercholesterolemia. The index case of one of these families was a 69 years old woman with arcus cornealis, evidence of premature coronary artery disease (angor at 56 years) and history of hypercholesterolemia in several family embers (two brothers with TC 700 and 435 mg/dL respectively). She was clinically diagnosed as having FH with a MedPed score of 13 points. Plasma lipid levels with lipid lowering treatment with simvastatin (40 mg/dL) were: TC 444 mg/dL, LDL-c 368 mg/dL and normal TG and HDL-c levels. After lipid lowering treatment with atorvastatin (30 mg/day) reduced her LDL-c to 225 mg/dL.

**EXAMPLE 13: Identification of mutations located in exon 13 of the LDLr gene.**

A 215 bp fragment of exon 13 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the following primers: Ex13F (SEQ ID NO: 27) y Ex13R (SEQ ID NO: 28

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 74°C for 3 min, and a final extension of 72°C for 10 min

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**D630N mutation analysis**

This mutation D630N (1951G>A, GAT>AAT, Asp630Asn) destroy a MnlI digestion site. Fifteen $\mu$L of PCR sample were digested with 15 units of MnlI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 89, 48, 39, 14+14 ,12 and 11 bp in normal alleles and 89, 59, 39, 14+14 and 12 bp in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126 y SEQ ID NO: 127

D630N mutation was detected in two unrelated families with autosomal dominant heredity. Index case of one of this family was a 36 years old woman whose parents died of myocardial infartion at 62 and 64 years of age. The MedPed score for FH clinical diagnostic was 7 points. The plasmatic lipid concentrations without pharmalogical treatment were: TC (332 mg/dL) and LDLc (268 mg/dL), TG (81mg/dL) and HDLc (48 mg/dL).

**H635N mutation analysis**

As this mutation H635N (1966C>A, CAC>AAC, His635Asn) does not change the restriction map, a desoxyoligonucleotide with two mismatches was designed and synthetized to introduce the recognition site of CaiI in presence of the normal allele and disappearing in the prsence of the mutant allele.

A 169 bp fragment of exon 13 was amplified the PCR technique using the desoxyoligonucleotide Ex13F (SEQ ID NO: 27) and the desoxyoligonucleotide with two mismatches MutH635NR (SEQ ID NO: 29).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

Fifteen μL of PCR sample were digested with 15 units of CaiI in a total volume of 30 μL according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 151 and 18 bp in normal alleles and 169 bp in mutant alleles. These fragments were electrophoresed in 8% PAA gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130 y SEQ ID NO: 131

H635N mutation was detected in a member of autosomal dominant hypercholesterolemia family. The subject was a 43 years old man with arcus corneae and without a prematuer cardovascular disease family history. His mother and three siblings had cholesterol concentrations above the 95 percentile. He was clinically diagnosed as having FH with a MedPed score of 13 points. His plasmatic lipid concentrations without pharmacological treatment were: TC (448 mg/dL) and LDLc (384 mg/dL) with normal TG and HDLc levels.

**EXAMPLE 14: Identification of mutations located in exon 14 of the LDLr gene.**

A 288 bp fragment of exon 14 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the desoxyoligonucleotides Ex14F (SEQ ID NO: 30) and Ex14R (SEQ ID NO: 31).

DNA (250ng) was amplified in a 50 μL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 20 μM each dNTP, 0.2 μM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA,

USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip".

**D686Ymutation analysis**

This mutation D686Y (2119G>T, GAC>TAC, Asp686Tyr) was identified by automatic of 288 bp fragment from exon 14 of the LDL-r gene on analyzing this fragment in subjects clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex14F (SEQ ID NO: 30) and Ex14R (SEQ ID NO: 31).

The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The change observed G>T was confirmed by automatic sequencing of a second PCR product of the same sample. Alternatively, this mutation can be analysed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 216, SEQ ID NO: 217, SEQ ID NO: 218 and SEQ ID NO: 219.

30) y Ex14R (SEQ ID NO:

D686Y mutation was detected in a 31 years old man with xantomas, arcus corneae, evidence of premature coronary artery disease (angor) and family history of hypercholesterolemia. He was diagnosed clinically as having FH with a MedPed score of 21 points. His plasmatic lipid concentrations prior to pharmacology treatment were: TC (430mg/dL) with normal TG and HDLc levels. After combined lipid lowering treatment with atorvastatin 40mg/day and colestipol 5 (g/day) the TC level decreased to 205 mg/dL.

**EXAMPLE 15: Identification of mutations located in exon 15 of the LDLr gene.**

A 243 bp fragment of exon 15 of the LDLr gene was amplified by polymerase chain reaction (PCR) using the desoxyolygonucleotides Ex15F (SEQ ID NO: 32) and Ex15R (SEQ ID NO: 33).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 20 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification

cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 seconds, and elongation at 72°C for 1.5 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip"..

**2184delG mutation analysis**

This mutation creates a new cleavage for the restriction enzyme for AluI.. Fifteen $\mu$L of PCR sample were digested with 15 units of AluI in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Gibco BRL, Carlsbad, CA, USA). The fragments obtained had a length of 166 and 78 bp for normal alleles and 166, 67 and 11 bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134 y SEQ ID NO: 135

2184delC mutation was detected in an autosomal dominant hypercholesterolemia family. The subject was a 32 years old woman, with family history of premature cardiovascular disease. The MedPed score for FH clinical diagnostic was 6 points. The plasmatic lipid concentrations without pharmacological treatment were: TC (330 mg/dL) and LDLc (270 mg/dL) with normal TG and HDLc levels.

**Aanalysis of the T740M mutation**

This mutation T740M (2282C>T, ACG>ATG, Tyr740Met) creates a new NlaIII digestion site. Fifteen $\mu$L of PCR sample were digested with 15 units of NlaIII in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (NEB, Beverly, MA, USA). The fragments obtained had a length 247 pb for normal alleles and 274, 194

y 53 bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 192, SEQ ID NO: 193, SEQ ID NO: 194 and SEQ ID NO: 195.

T740M mutation was detected in a 60 years old woman, with arcus corneae, family history of hypercholesterolemia and family history of premature cardiovascular disease. Father died at 34 years with cerebralvascular incident. She has been diagnosed as having familial hypercholesterolemia with a MedPed diagnostic score of 10 points. The plasmatic lipid concentrations prior to pharmacology trreatment were: TC 492 mg/dL and normal TG and HDL-c levels. After lipid lowering treatment with atorvastatin the TC level lowered to 251 mg/dL

**EXAMPLE 16: Identification of mutations located in exon 16 of the LDLr gene.**

A 273 bp fragment of exon 16 was amplified by polymerase chain reaction (PCR) using the following primers: Ex16F (SEQ ID NO: 152) and Ex16R (SEQ ID NO: 153).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 20 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described

previously "biochip

**V766E mutation analysis**

This mutation V766E (2360T>A, GTG>GAG, Val766Glu) was identified by automatic sequencing of the 273 pb fragment from exon 16 of the LDL-r gene on analyzing this fragment in patients clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Beckman Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex 16F (SEQ ID NO: 152) y EX16R (SEQ ID NO: 153). The fragments generated by the sequencing reaction were analyzed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The change T>A change observed was confirmed by sequencing a second PCR product. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 236, SEQ ID NO: 237, SEQ ID NO: 238 y SEQ ID NO: 239.

D686Y mutation was detected in a 58 years old woman with tendon xantomas in elbows, arcus corneae, xantelasmas and family history of hypercholesterolemia. She was diagnosed clinically as having FH with a MedPed score of 12 points. The plasmatic lipid concentration prior to pharmacological treatment were: TC (420mg/dL), LDL-c (324 mg/dL) with normal TG and HDLc levels.

**I771T mutation analysis**

As this mutation I771T (2375T>C, ATT>CACT, Ile771Thr), does not change the restriction map, a mismatched desoxyoligonucleotide was designed and synthetized to introduce the recognition site of HincII in presence of the mutant allele and disappearing in the presence of normal allele.

A 142 bp fragment of exon 16 of the LDLr gene was amplified by the PCR technique using desoxyoligonucleotide Ex16R (SEQ ID NO: 153) and the mismatched desoxyoligonucleotide MutI771TF (SEQ ID NO: 154).

DNA (500ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

Fifteen $\mu$L of PCR sample were digested with 15 units of HincII in a total volume of 30 $\mu$L according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 142 bp in normal alleles and 121 and 21 bp in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198 y SEQ ID NO: 199.

I771T mutation was detected in a 60 years old woman with evidence of premature coronary disease in the family and hypercholesterolemia family history. She has been diagnosed as having familial hypercholesterolemia with a MedPed diagnostic score of 21 points. Her plasma lipid levels were: TC (422 mg/dL) and LDLc (368 mg/dL), and normal TG and HDLc levels

**2389+3 A>C mutation analysis**

This mutation 2389+3 C>T was identified by DNA sequencing of the 273 pb fragment from exon 16 of the LDL-r gene in subjects clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex16F (SEQ ID NO: 152) and Ex16R (SEQ ID NO:153). The fragments generated by the sequencing reaction were analysed in an automatic sequencer CEQ 2000XL DNA Beckman Analysis System. The change observed C>T was confirmed by automatic sequencing of a second PCR product of the same sample.

Alternatively, this mutation can be analysed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 252, SEQ ID NO: 253, SEQ ID NO: 254 and SEQ ID NO: 255.

2389+3 C>T mutation was detected in a 36 years old man with aquiles heel tendon xantomas and hand extenders and history of hypercholesterolemia in the family (mother, brother and one son with LDL-c levels above the 95 percentile). He was diagnosed clinically as having FH with a MedPed score of 18 points. The plasmatic lipid concentrations before pharmacological treatment were: TC (450mg/dL) with normal TG and HDLc levels. Lipid lowering treatment with atorvastatin (20mg/dL) reduced his LDL-c to 259 mg/dL

**2389+4 A>G mutation analysis**

As this mutation (2389+4 A>G) does not change the restriction map, a mismatched desoxyinucleotide was designed and synthetized to introduce the recognition site of BshNI in presence of the mutant allele but not in presence of normal allele.

A 194 bp fragment of exon 16 gene was amplified by polymerase chain

reaction (PCR) using the following primers: Ex16F (SEQ ID NO: 152) and the mismatched desoxyioligonucleotide Mut2389+4 A>GR (SEQ ID NO: 155).

DNA (500ng) was amplified in a 50 μL reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 μM each dNTP, 0.2 μM each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 10 min.

Fifteen μL of PCR sample were digested with 15 units of BshNI in a total volume of 30 μL according to the protocol described by the manufacturer (Fermentas Inc., Hanover, MD, USA). The fragments obtained had a length of 194 bp for normal alleles and 175 and 19 bp for mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed with the device described ("biochip") by spotting onto the slide the oligonucleotides SEQ ID NO: 180, SEQ ID NO: 181, SEQ ID NO: 182 y SEQ ID NO: 183..

2389+4 A>G mutation was detected in 11 unrelated hypercholesterolemic families. Index case of one of this families was a 22 years old woman with tendon xanthomas and family history of premature cardiovascular disease (father with hypercholesterolemia and myocardial infartion at age 29). She has been diagnosed as having familial hypercholesterolemia with a MedPed diagnostic score of 17 points. Her plasma lipid levels without lipid lowering treatment were: TC (356 mg/dL) and LDLc (293 mg/dL), and normal TG and HDLc levels. Combined lipid lowering treatment with atorvastatin (40 mg/day) and colestipol (5g/day) lowered her HLD-c level to 227 mg/dL

**EXAMPLE 17: Identification of mutations located in exon 17 of the LDLr gene.**

A 242 bp fragment of exon 17 was amplified by polymerase chain reaction (PCR) using the following primers: Ex17F (SEQ ID NO: 34) and Ex17R (SEQ ID NO: 35).

DNA (300ng) was amplified in a 50 $\mu$L reaction mixture containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCL_2$, 200 $\mu$M each dNTP, 0.2 $\mu$M each primer and 1.5 units of Taq DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The amplification cycle was: 10 min of denaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min, and a final extension of 72°C for 10 min.

PCR products were analyzed by Single Strand Conformation Polymorphisms (SSCP). Those fragments that showed abnormal SSCP patterns were sequenced using an automated CEQ 2000XL DNA Analysis System (Beckman Coulter, Palo Alto, CA, USA). The presence of the identified mutations was subsequently determined by restriction analysis and by the device described previously "biochip

**2399del5ins4 mutation analysis**

This mutation eliminate the sequence TCTTC and introduces the sequence GGGT at the 2399 position, and creates a new AvaI digestion site. Fifteen μL of PCR sample were digested with 15 units of AvaI in a total volume of 30 μL according to the protocol described by the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The fragments obtained had a length of 230 and 12 bp in normal alleles and 183, 46 and 12 bp in mutant alleles. These fragments were electrophoresed in 8% polyacrilamide gel and were visualized by staining with ethidium bromide. Alternatively, this mutation could be analyzed by the device described ("biochip") using into the slide the oligonucleotides SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 138 and SEQ ID NO: 139.

2399del5ins4 mutation was detected in three unrelated hypercholesterolemic families with autosomal dominant inheritance. The index case of one of these families was a 49 years old woman, with tendon xanthomas whose father had died at 51 with myocadial infartion.. The MedPed score for FH clinical diagnostic was 16 points. Her plasma lipid levels before lipid lowering treatment were: TC (510 mg/dL, LDLc (424 mg/dL).), HDLc (58 mg/dL) and TG(140 mg/dL). Combined treatment with simvastatin 20 mg/day and colestipol 20 g/day lowered her TC to 280 mg/dL. Furthermore, two children of hers, aged 22 and 20 years, had cholesterol levels of 330 and 386 mg/dL respectively.

**2544insC mutation analysis**

This mutation was identified by automatic sequencing of the 242 pb fragment from exon 17 of the LDL-r gene on analyzing this fragment in subjects clinically diagnosed as having FH. The sequencing reaction was performed in a thermocycler PE Gene Amp System 9700 using the kit CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start (Beckman Coulter, Palo Alto, CA, USA) and the primers Ex17F (SEQ ID NO: 34) and Ex17R (SEQ ID NO: 35), the subsequent electrophoresis in automatic sequencer CEQ 2000 DNA Beckman Analysis System. This deletion was confirmed by automatic sequencing of a second PCR product from the same sample.

Alternatively, this mutation can be analysed with the device described ("biochip") by spotting onto the slide the oligonucleotides: SEQ ID NO: 244, SEQ ID NO: 245, SEQ ID NO: 246 and SEQ ID NO: 247.

2544insC mutation was detected in a 37 years old man who had suffered a myocardial infartion and with tendon xanthomas, arcus corneae, family history of hypercholesterolemia (his father died prematurely of mycardial infartion). He was diagnosed clinically as having FH with a MedPed score of 21 points. The plasmatic lipid concentrations prior to pharmacology treatment were: TC (444 mg/dL), LDL-c (379 mg/dL) with normal TG and HDLc levels. Lipid lowering treatment with atorvastatin (40mg/dL) lowered his LDL-c to 282 mg/dL

**Description of the drawings**

**Figure 1** is a schematic representation of the itinerary of the LDL-r in human cells. The LDL-r is synthesized in the endoplasmic reticulum as a precursor of apparent molecular weight of 120 Kd and transported to the Golgi apparatus. Once transferred to the surface of the cell the receptor recognizes the apolipoprotein B-100 component of the LDL. Binding leads to cellular uptake and lysosomal degradation of the LDL by receptor-mediated endocytosis. This uptake process satisfies the cholesterol needs of the cells, and hence keeps endogenous cholesterol synthesis suppressed.

**Figure 2** is a schematic representation of the five domains in the structure of the human LDL receptor protein and their correspondence with the gene exons.

**Figure 3** Glass slide for quantification of image with 4 primers (2 normal and 2 mutated) repeated in 10 cups for the mutation E256K. (A) normal individual (B) individual with familial hypercholesterolemia. Two pairs of oligonucleotides were spotted for each mutation. Each probe pair consists of one probe specific for the wild-type allele and a second probe specific for the mutant allele.